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Synthesis of C3, C5, C6, C7, C8, and C9 by Human Fibroblasts

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We investigated the ability of human fibroblasts to produce the components of the final common pathway (C3–C9) of complement in vitro by co-culturing an alternative complement activator (agarose beads) with the cells. The test system involved incubation of beads with anti-complement antibodies followed by radioactive-labelled anti-Ig detection antibodies. Subsequently, the beads were examined in a radioimmunoassay. Our results indicate that human fibroblasts produce C3, C5, C6, C7, C8, and C9. A neoepitope selectively expressed on activated C9 was detected, indicating assembly of the terminal complement complex and thus formation of a functional terminal complement pathway by the fibroblasts.

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Complement is an integral part of host defence against invading microorganisms and of the inflammatory process. The system is a complicated network composed of about 25 plasma and membrane-bound proteins that interact upon activation in a precise and highly regulated manner [3]. The major source of the complement proteins is the liver, although numerous investigations have shown that extrahepatic synthesis of complement proteins occurs [2]. These studies indicate the potential of locally produced complement as microbicidal factors and inflammatory mediators. Moreover, enhanced tissue-specific expression of complement genes at inflammatory sites suggests that local production and activation of complement proteins may play a role in the immunopathology of certain autoimmune diseases [16].

Many different extrahepatic cell types have been shown to produce complement components from the classical and alternative pathway including monocytes, endothelial cells, astrocytes, epithelial cells, and fibroblasts [1, 11, 13, 25]. Synthesis of the terminal complement components (C5–C9) has been detected in monocytes [9]. Recently, pneumocytes have also been shown to produce terminal complement proteins [18].

We here report that human fibroblasts are able to produce components of the final common pathway (C3–C9). Assembly of the terminal complement complex (TCC) on the surface of agarose beads, a potent alternative complement pathway activator, indicates that the proteins were functionally active.

MATERIAL AND METHODS

Cells. The human embryonal fibroblast cell lines were obtained from Flow laboratories, Irvine, UK (MRC-5) and from our own laboratory (He 9). The cells were grown to confluence in 16-mm Costar culture wells (Costar, Cambridge, Mass., USA) in Dulbecco's modified Eagle's medium (DMEM) with glutamine (200 mM), $NaHCO_3$ (2.0 g/l), and 2% inactivated fetal calf serum, and incubated at 37°C in air with 5% CO_2. Some cells were cultured overnight with 1 μg/ml lyophilized endotoxin (ET) (*Escherichia coli* 026; B6, Difco Lab., Detroit, Mich., USA). All fibroblast cultures were washed (×4) before the experiments were performed under serum-free conditions in DMEM also containing penicillin (105 IU/l) and streptomycin (0.1 g/l). One milligram (5 × 104) of large Sepharose 4B (S4B) agarose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) was incubated with each cell culture well or medium for 24 or 48 h in the absence or presence of 25 μCi/ml l-[^{3}H] leucine (sp. act. 110 Ci/mmol; New England Nuclear, Dreireich, FRG) or 1 or 5 μg/ml cycloheximide (Sigma, St Louis, Mo., USA), or with normal human serum for 1 h.

Undiluted supernatants (0.5 ml) were harvested after 24 h and 48 h from cell cultures incubated with or without S4B beads. The beads were then removed by a Pasteur pipette and centrifuged for 5 min at 50 *g* through a 5-ml layer of Lymphoprep (Nycomed, Oslo, Norway) in conical tubes and washed twice in phosphate-buffered saline containing 2 mg/ml bovine serum albumin (PBSA) to remove protein unspecifically bound to the beads before agarose-bound radioactivity was recorded. Control beads were treated similarly. The binding index ± 1 standard deviation of labelled protein to the basis is defined as: counts per minute (cpm) of beads kept in cell cultures with [^{3}H]leucine divided by cpm of beads kept in medium with [^{3}H]leucine.

Preparation of antibodies. Highly specific goat antisera to human C6 and C7 was an appreciated gift from Dr H.J. Müller-Eberhard (Scripps Clinic, La Jolla, Calif., USA). The antibody specificities have previously been described in detail [9]. Rabbit anti-C3d and anti-C5 were obtained from Dakopatts, Copenhagen, Denmark and rabbit anti-C3c and anti-C9 from Behringwerke, Marburg, FRG, and tested as described [4]. Goat anti-C8 was purchased from Atlantic Antibodies Inc., Scarborough, Maine, USA. Monoclonal antibody bH6 against a neoepitope on C3b, iC3b, and C3c and monoclonal antibody aE11 reacting with a neoepitope in C9 were produced in our own laboratory [6, 15]. ^{125}I-labelled sheep anti-mouse Ig and donkey anti-rabbit Ig, and peroxidase-labelled sheep anti-mouse Ig and donkey anti-rabbit Ig were purchased from Amersham, Buckinghamshire, UK. Rabbit anti-goat Ig was obtained from Dakopatts and goat anti-rabbit Ig that was used as irrelevant antibody from Vector Lab., Burlingame, Calif., USA.

Binding of antibodies to the agarose beads. Anti-complement antibodies diluted in appropriate dilutions were incubated for 30 min at 37 C with the S4B beads (5×10^4) that had been preincubated for 24 or 48 h with the fibroblast cultures and washed in PBSA. In experiments with goat anti-C6, C7, and C8 rabbit anti-goat Ig were used in a second step. [^{125}I]labelled anti-Ig antibodies were added after further washing before testing in the radioimmunoassay. Controls were co-cultured beads incubated with BSA or an irrelevant antibody instead of anti-complement antibodies. Control experiments were also performed with agarose beads incubated in normal human serum for 1 h.

Radioimmunoassay (RIA). Detection of complement bound to agarose beads in RIA was performed in general as previously described [8]. Binding index ± SD of the antibodies to the beads is defined as: cpm of beads treated in cultures or normal serum and incubated with primary and with or without secondary antibodies and ^{125}I-labelled anti-Ig divided by cpm of beads treated in cultures or serum and incubated with BSA or an irrelevant antibody and ^{125}I-labelled anti-Ig.

Analyses of fluid phase complement activation products. Complement activation in concentrated culture supernatants (10×) were assessed in two enzyme immunoassays (EIA) specific to neoepitopes on activation products not expressed in native complement factors. One was specific for C3 activation products, thus evaluating the initial part of the complement cascade [5]. The other assay detected the fluid phase TCC [14].

Examination of C3, C5, C9, and activated C9 (C9neo) in SDS-PAGE and in solid-phase EIA. Concentrated culture supernatants were tested in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent western blot analysis [12, 21]. Detection antibodies were polyclonal anti-C3d, anti-C5, and anti-C9. Furthermore, solid-phase C3, C5, C9, and TCC EIA were constructed. Concentrated supernatants were diluted 1:2 and adsorbed to polystyrene wells (Teknunc, Copenhagen, Denmark) overnight at room temperature antibodies in appropriate concentrations were then added. Subsequently, the reaction was processed with peroxidase-labelled anti-rabbit or anti-mouse antisera.

Statistics. The significance of the RIA results was tested by Student's *t* test. *P* values below 0.05 were considered to be statistically significant. In the solid-phase EIA experiments background +2 standard deviation (+2SD) was considered significant.

RESULTS

Binding of ^{3}H-labelled protein and anti-C3 antibodies to S4B beads incubated with fibroblasts

In control experiments there was a specific, and by cycloheximide reducible, binding of tritium-labelled protein to the co-cultured beads (Table I).

Since detection of fibroblast-derived C3, as agarose-bound C3b, is prerequisite for the generation of particle-bound C5 convertase, we tested the binding of anti-C3 antibodies to agarose beads with both the MRC-5 and He 9 cell lines. A significant amount of C3 was found on agarose beads harvested from cultures of both cells after 24 or 48 h of incubation (Table II). Prolonged incubation (48 h) increased the binding index of C3 to the beads. Synthesis of C3 was inhibited by addition of cycloheximide (Table II) or ET (data not shown) to the cell cultures.

Agarose binding of antibodies to the terminal components

To study deposition of the terminal complement components on co-cultured S4B beads, polyclonal antibodies against C5, C6, C7, C8, C9 and a monoclonal antibody against a C9 neoepitope were used (Tables III and IV). S4B beads incubated with the MRC-5 cells were shown to bind all the terminal components except C8, indicating novel synthesis of these components. Cycloheximide inhibited the synthesis of the complement proteins after 24 h (inhibition of C6

TABLE I. Internal labelling of protein. De novo synthesis by MRC-5 and He 9 fibroblast cell lines of protein with affinity for S4B agarose beads

S4B beads kept with	Incubation time (h)	Binding of ^{3}H-labelled protein to beads co-cultured with fibroblasts	
		−cycloheximide	+cycloheximide*
MRC-5	24	22.9 ± 0.8	7.5 ± 0.5
MRC-5	48	40.8 ± 0.4	18.1 ± 3.9
He 9	24	24.0 ± 1.3	5.3 ± 0.3
He 9	48	59.5 ± 4.2	14.5 ± 2.4

The results are given as the mean ± SD of three experiments. The binding index is defined as: cpm of beads kept in the cell cultures + [^{3}H]leucine divided by cpm of beads kept in medium + [^{3}H]leucine (115 ± 14).
*5 μg/ml.

TABLE II. Detection by RIA of C3 on S4B beads incubated with MRC-5 and He 9 fibroblast cultures or serum

S4B beads kept with	Incubation time (h)	Addition of	Binding index of [^{125}I]anti-Ig to beads incubated with cells or serum and then with	
			Anti-C3c	Anti-C3d
MRC-5	24	—	7.1 ± 1.1	2.5 ± 0.6
MRC-5	24	Cyclo	ND	NS
MRC-5	48	—	14.1 ± 0.4	ND
He 9	24	—	7.8 ± 1.6	NS
He 9	24	Cyclo	ND	NS
He 9	48	—	13.2 ± 0.6	ND
Serum	1	—	102.4 ± 7.1	37.7 ± 4.2

The results are given as the mean ± SD of at least two experiments. Binding index: cpm of beads treated in cultures or normal serum and incubated with primary antibody and anti-Ig divided by cpm of beads treated in cultures or serum and incubated with BSA and anti-Ig (range 2022–3964 cpm).
Cyclo, cycloheximide; ND, not determined; NS, not significant.

was observed after 48 h). Moreover, when ET was added no significant synthesis of the components was observed (data not shown). In contrast to the situation with MRC-5, only C6 and C9 were detected on the beads incubated with He 9 cells.

To investigate whether the bound complement components were assembled into TCC, we examined the binding of a monoclonal antibody (aE11) specifically reactive with a neoepitope in polymerized C9 (Table IV). We observed a significant anti-C9neo antibody binding to beads incubated with the MRC-5 cells. In contrast, no binding of the antibody was observed with beads co-cultured with He 9 cells.

Incubation of S4B beads in serum for 1 h showed binding of C3 and all the terminal components.

Analyses of fluid-phase components

No soluble complement components were found either in fluid-phase EIA, SDS–PAGE, or in solid-phase EIA.

TABLE III. Detection by RIA of C6, C7, and C8 on S4B beads incubated with MRC-5 and He 9 fibroblast cultures or serum

S4B beads kept with	Incubation time (h)	Addition of	Binding index of [^{125}I]anti-Ig to beads incubated with cells or serum and then with rabbit anti-goat Ig and subsequently with: Anti-C6	Anti-C7	Anti-C8
MRC-5	24	—	1.8±0.0	NS	NS
MRC-5	24	Cyclo	1.8±0.0	NS	NS
MRC-5	48	—	2.0±0.2	2.3±0.2	NS
MRC-5	48	Cyclo	NS	NS	NS
He 9	24	—	NS	NS	NS
He 9	24	Cyclo	ND	ND	ND
He 9	48	—	2.3±0.2	NS	NS
He 9	48	Cyclo	NS	ND	NS
Serum	1	—	2.9±0.4	3.0±0.6	2.6±0.1

See footnotes to Table II. The range of the cpm values for control beads treated in cultures or serum and incubated with BSA and anti-Ig was 2544–3481.

TABLE IV. Detection by RIA of C5, C9, and TCC on S4B beads incubated with MRC-5 and He 9 fibroblast cultures or serum

S4B beads kept with	Incubation time (h)	Addition of	Binding index of ^{125}I-anti-Ig to beads incubated with cells or serum and then with: Anti-C5	Anti-C9	aE11
MRC-5	24	—	1.9±0.2	4.9±0.6	2.7±0.1
MRC-5	24	Cyclo	NS	NS	ND
MRC-5	48	—	1.9±0.4	ND	2.0±0.1
MRC-5	48	Cyclo	ND	ND	ND
He 9	24	—	NS	3.7±0.2	NS
He 9	24	Cyclo	NS	NS	ND
He 9	48	—	NS	ND	NS
Serum	1	—	7.0±1.3	26.0±4.4	8.2±0.2

See text to Table II. The range of the cpm values for control beads treated in cultures or serum and incubated with BSA and anti-Ig was 2544–3481.

DISCUSSION

From the present study we conclude that human fibroblasts in vitro synthesize and secrete the complement components C3, C5, C6, C7, and C9 as detected on co-cultured agarose beads by anti-C3, anti-C5, anti-C6, anti-C7, and anti-C9. Furthermore, binding of an antibody specifically recognizing a C9 neoepitope implies that TCC is formed on the beads. This latter finding indicates that C8 may also be synthesized, since C5b-9 is not formed without generation of C5b-8, although we were not able to detect C8 directly.

De novo synthesis of ^{3}H-labelled protein with affinity for the complement-activating beads was inhibited by cycloheximide. We exclude the possibility that our results are the result of secretion of endocytosed complement, since cycloheximide abrogated detection of complement on the beads. C6 was detected on the beads co-cultured with the MRC-5 cell line in the pre
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MRC-5 cell line in the presence of cyclo-heximide after 24 h. However, prolonged incubation (48 h) revealed de novo synthesis of C6 (Table III).

Lipopolysaccharide (LPS) has been demonstrated to induce inflammation, to activate the complement system, and to regulate complement synthesis in monocytes [2]. In the present study we found that ET, which are LPS–protein complexes, inhibited secretion of the complement components in both cell lines. Recently, LPS has been shown to both stimulate and inhibit complement synthesis in fibroblasts [10, 19].

For unknown reasons we failed to detect the components in the culture supernatants. It may be possible that secretion of the terminal components from the fibroblasts may require direct contact with other cells or particles. This has been observed for perforin, a molecule partly homologous to C9 which is secreted from MHC-restricted class I cytotoxic T lymphocytes and NK cells [22, 23]. Alternatively, the terminal components and C3 may have been absorbed to the agarose beads and depleted from the fluid phase.

Fibroblasts are abundant and widely distributed in most tissues. The major secretory products are elastin, collagen, and proteoglycans [7]. When stimulated, they may express MHC class II molecules and present antigens to lymphocytes [17, 24]. Moreover, synthesis of complement proteins from both the alternative and classical pathway has been documented in these cells [10, 20].

This study demonstrates that human fibroblasts also produce a functional terminal complement pathway. Fibroblasts may thus serve as a local regulator or the inflammatory reaction in tissues.

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SHORT PAPER

Fcγ Receptor
Placenta

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